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Enhancement of Hepatitis C Virus NS3 Proteinase Activity by Association with NS4A-Specific Synthetic Peptides: Identification of Sequence and Critical Residues of NS4A for the Cofactor Activity

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The NS3 proteinase of hepatitis C virus utilizes NS4A as a cofactor for cleavages at four sites (3/4A, 4A/4B, 4B/5A, and 5A/5B) in the nonstructural region of the viral polyprotein. To characterize NS4A for its role in modulating the NS3 proteinase activity at various cleavage sites, synthetic peptides spanning various parts of NS4A were synthesized and tested in a cell-free *trans*-cleavage reaction using purified NS3 proteinase domain and polyprotein substrates. The NS3 proteinase domain was expressed in *Escherichia coli,* purified, denatured, and refolded to an enzymatically active form. We found that a 12-amino-acid peptide containing amino acid residues 22 to 33 in NS4A (CVVIVGRIVLSG) was sufficient for cofactor activity in NS3-mediated proteolysis. The peptide enhanced the cleavage at the NS5A/5B site and was necessary for NS3-mediated cleavage at NS4A/4B and NS4B/5A. Sequential amino acid substitution within the designated peptide identified residues I_{29} and I_{25} as critical for potential cofactor activity. We provide evidence that the NS4A peptide and the NS3 catalytic domain form an enzymatically active complex. These data suggest that the central 12-amino-acid peptide (aa 22–33) of NS4A is primarily important for the cofactor activity through complex formation with NS3, and the interaction may represent a new target for antiviral drug development.

INTRODUCTION

Hepatitis C virus (HCV), the major etiological agent of posttransfusion non-A non-B hepatitis (Choo *et al.,* 1989; Kuo *et al.,* 1989), is classified as a member of the flavivirus family (Miller and Purcell, 1990). Several genotypes of HCV, each with many strains, have been identified (Simmonds *et al.,* 1993). HCV contains a positive-sense linear RNA genome of approximately 9.5 kb which encodes a single large polyprotein precursor (Choo *et al.,* 1991; Takamizawa *et al.,* 1991). Although it is yet to be confirmed by infectivity assay, cell-free translation of the viral RNA (Hijikata *et al.,* 1991) and cell culture expression studies (Grakoui *et al.,* 1993a,b; Hijikata *et al.,* 1993) have established that the HCV polyprotein is proteolytically processed by host and viral proteinases into 10 different proteins which are encoded as follows: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. The proteins encoded within the amino-terminal one-third, C, E1, E2, and p7, have been reported to be proteolytically processed into mature forms by host signalases (Hijikata *et al.,* 1991; Lin *et al.,* 1994). The remainder of the polyprotein is proteolytically processed by two virally encoded proteinases, NS2-3 and NS3, to produce mature proteins NS2 through NS5B. The cleavage between NS2 and NS3 is accomplished intramolecularly by a poorly characterized viral metalloproteinase encoded within NS2 and the NS3 proteinase domain (Hijikata *et al.,* 1993; Grakoui *et al.,* 1993c; Reed *et al.,* 1995). NS3 proteinase mediates the rest of the cleavages in the nonstructural part of the polyprotein, with processing between NS3 and NS4A occurring *in cis,* and cleavages between 4A/4B, 4B/5A, and 5A/5B occurring *in trans.* This proteinase activity has been mapped to the amino-terminal 181 aa residues of NS3. It is a member of the serine proteinase family and contains serine, histidine, and aspartic acid as a catalytic triad, serine being the active site residue (Grakoui *et al.,* 1993b; Tomei *et al.,* 1993). It has been shown that NS4A enhances the cleavage at the 5A/5B site and is absolutely required for cleavage at the 4B/5A site (Failla *et al.,* 1994; Lin *et al.,* 1995). NS4A is cleaved off from NS3 by intramolecular cleavage at NS3/4A site and has been shown to enhance NS3 activity *in cis* and *in trans* (Failla *et al.,* 1994). It is a 54-amino-acid residue protein and the central region has been reported to be important for the cofactor activity (Lin *et al.,* 1995). In addition to its role in modulating NS3-mediated proteolysis, NS4A has also been reported to enhance phosphorylation of NS5A (Tanji *et al.,* 1995). NS4A is a multifunctional protein and

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the mechanisms by which it enhances NS3 proteinase activity and phosphorylation of NS5A are not known.

Understanding the precise mechanism of NS4A in modulating NS3 activity is important, not only for physical and biochemical characterization of the enzyme, but also for the design of specific inhibitors which might be developed as potential antiviral drugs. In this report, we examine the nature of the NS4A cofactor activity in a biochemical *trans*-cleavage reaction using purified NS3 proteinase, NS4A synthetic peptides, and *in vitro*-translated polypeptides as substrates. We have identified a 12-aa residue NS4A-specific synthetic peptide (aa 22–33) as the minimum sequence necessary to modulate the NS3-mediated proteolytic activity and have found by alanine substitution that the hydrophobic amino acids in the peptide are important for the cofactor activity. We show that the same peptide of NS4A increases the efficiency of cleavage at the 5A/5B site and is absolutely required by the NS3 proteinase for the cleavages at the 4A/4B and 4B/5A sites. We demonstrate for the first time that the peptide forms an enzymatically active complex with the NS3 proteinase domain.

MATERIALS AND METHODS

Construction of plasmid DNAs for expression of the NS3 proteinase domain and polypeptide substrates

The plasmid pBRTM/HCV 1-3011 was used as the starting material and the nucleotide numbers indicated refer to the HCV sequence in the plasmid pBRTM/HCV 1-3011 (Grakoui *et al.,* 1993a). A DNA fragment from nucleotide 7607 to 8344, encoding the amino-terminal 246 amino acids of NS3, was isolated after several subcloning procedures and was cloned into *Asp*I-digested, Klenow polymerase-treated pQE30 (HIV) (B. DasMahapatra, personal communication) to produce the plasmid pBJ1015. A translational stop codon after amino acid 183 in the NS3 sequence in plasmid pBJ1015 was engineered by inserting the oligonucleotide

GA TCA CCG GTC TAG ATC T

T GGC CAG ATC TAG A

after nucleotide 8144 in the HCV genome. The resulting plasmid, His-HIV-NS3(183), expresses a fusion protein containing a polyhistidine (6× His) tag for purification, an 11-aa peptide containing the HIV-1 protease cleavage site to remove the purification tag if necessary, and the amino-terminal 183 aa of NS3. The construction was verified by nucleotide sequencing. The bacterially expressed NS3 catalytic domain was purified from inclusion bodies, denatured with 6 *M* guanidine–HCl, and refolded into enzymatically active form with detergent. The preparation was greater than 95% pure. The amino-terminal 15 aa residues of the fusion protein were confirmed by amino acid sequence determination. Detailed methods of *Escherichia coli* expression, purification, and refolding of the His-HIV-HCV proteinase domain will be described elsewhere (Ramanathan *et al.,* 1996).

To produce HCV NS3 substrate polypeptides with different cleavage sites, DNA fragments from the plasmid pBRTM/HCV encoding regions of nonstructural polyprotein were isolated and cloned into the cell-free expression plasmid pBD7 (DasMahapatra, 1993). The HCV coding sequences in these plasmids are in frame with the initiator ATG of pBD7. The plasmid pAH 21 contains HCV sequences from 9474 through 13,610; the plasmid pNBNae contains nucleotides 9604 to 10,238. Similarly the plasmids pJB1003 and pTS102 contain HCV sequences from 9798 to 13,606 and from nucleotides 11,462 to 13,190, respectively. Nucleotide numbers correspond to the numbers in the plasmid pBRTM/HCV 1-3001 (Grakoui *et al.,* 1993a). Restriction enzymes were purchased from New England Biolabs, Inc. DNA manipulations and other recombinant techniques were performed according to the *Molecular Cloning Manual* (Sambrook *et al.,* 1989).

Cell-free transcription and translation

The plasmid DNAs encoding HCV polypeptides with cleavage sites were linearized with appropriate restriction enzymes prior to transcription with T7 RNA polymerase. The plasmid pAH21 was linearized with *Bsu*36I and transcribed (DasMahapatra, 1993) to produce RNA encoding HCV polyprotein (NS3′-4A-4B′) from amino acid residue 1650 through amino acid residue 1956. Similarly, the transcript derived from the *Eco*RI-linearized plasmid pNBNae encodes the HCV protein (NS4A′-4B′) from aa residue 1693 to 1903. The RNA produced from the plasmid pJB1003 after linearizing with *Avr*II encodes HCV protein (NS4B′-5A′) from aa 1757 to 2048, and the RNA transcribed from the *Eco*RI-linearized plasmid pTS102 codes for HCV protein (NS5A′-5B′) from aa 2312 to 2621. The *in vitro*-transcribed RNAs were translated in rabbit reticulocyte lysates (Promega) in presence of [^{35}S]methionine (Amersham) at 30° for 1 hr according to the supplier's recommendation. Translation reaction were terminated by adding DNase-free RNase (Boehringer Mannheim) and cycloheximide (Sigma) to 10 μg/ml, followed by incubation for 15 min at 30°.

Cell-free proteinase assay

Standard proteinase assays were initiated by the addition of 1 μM refolded proteinase to 2 μl ^{35}S-labeled translated substrate in a 20-μl reaction volume containing 10 m*M* Tris, pH 7.5, 175 m*M* NaCl, 7.5 m*M* DTT, 0.5% EDTA, 0.1% Tween 20, and 12% glycerol, followed by incubation at 30° for 1 hr. NS4A-specific peptides were added to the standard assay mixture prior to the incubation, and appropriate solvents were added to standard assay mixtures as controls. Cleavage reactions were terminated

by adding an equal volume of 2× Laemmli sample buffer and boiling for 3 min. Cleavage products were analyzed by SDS–15% PAGE (Laemmli, 1970) and autoradiography.

Peptide synthesis

The peptides containing NS4A sequence and its derivatives were synthesized using Fmoc chemistry on an ABI Model 431 A peptide synthesizer. The peptides were cleaved off the resin and deprotected following a standard TFA cleavage protocol. The peptides were purified on reverse-phase HPLC and confirmed by mass spectrometric analysis.

Complex formation between the NS4A peptide and purified His-NS3 proteinase

To study complex formation between the peptide and the proteinase, the 13-aa synthetic peptide (aa 22–34) and the mutant peptide ($I_{25} \rightarrow A$ and $I_{29} \rightarrow A$) were separately mixed with the NS3 fusion protein. The mixture was incubated with the Ni-NTA beads (histidine binding resin; Qiagen, Inc.) in a final buffer concentration of 40 m*M* sodium phosphate, pH 7.8, 0.2 *M* NaCl, 8 m*M* β-mercaptoethanol, 0.2% Tween 20, and 20% glycerol, at a proteinase to peptide molar ratio of 1:2.5. Bead-bound complexes were separated by centrifugation (12,000 rpm for 30 sec in a microcentrifuge), washed with wash buffer (50 m*M* sodium phosphate, pH 7.8, 0.3 *M* NaCl, 10 m*M* β-mercaptoethanol, 0.1% Tween 20, and 25% glycerol) to remove free peptides, and assayed for the cleavage of ΔNS4A-Δ4B substrate. Imidazole was added (final conc 150 m*M*) to a duplicate set of reaction samples to elute the histidine-tagged complex from the beads. Similarly, an additional control experiment was set up by incubating the 13-amino-acid peptide alone with the beads. The complex was then washed as previously described and assayed for activity in the presence of NS3 proteinase.

RESULTS

Cofactor activity of NS4A in NS3-mediated proteolysis

To study the role of NS4A on the NS3-mediated *trans*-cleavages at NS4A/4B, 4B/5A, and 5A/5B of HCV nonstructural polyprotein, a series of HCV polypeptides with specific cleavage site(s) were produced by a cell-free transcription–translation system and processed in a *trans*-cleavage reaction by NS3 proteinase with or without NS4A. Figure 1 shows a schematic representation of polyprotein substrates. The NS3 proteinase used in this study is described under Materials and Methods. For NS4A we began with the carboxyl-end 33-amino-acid peptide (aa 22–54 of NS4A) of the HCV-1b (BK) strain (Failla *et al.,* 1994). The *in vitro*-translated polypeptide substrates were incubated with the NS3 proteinase in the

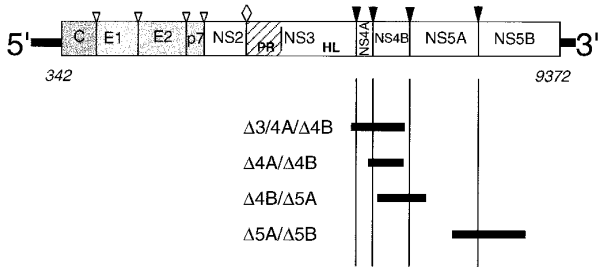

FIG. 1. Schematic representation of HCV genome and polyprotein. HCV NS3 proteinase catalytic domain used in the experiments is shown as a hatched box. The polyprotein regions used as *in vitro*-translated substrates are shown as solid black bars. Vertical lines indicate cleavage site positions relative to the precursor polyprotein.

presence or absence of the NS4A peptide. The results of the cell-free *trans*-cleavage reactions are shown in Fig. 2. The polypeptide containing the 3/4A and 4A/4B sites yielded an unprocessed precursor as well as two processed products with apparent molecular mass of 36, 29, and 7 kDa, respectively (Fig. 2, lanes 2 and 3). Processing was clearly evident by NS3 with or without NS4A; however, efficiency of the cleavage was improved in the presence of the NS4A peptide (compare lanes 3 and 2). From estimating the relative sizes of cleavage products we identified the larger product as Δ4B and the smaller as either ΔNS3/4A or 4A, indicating cleavage at 4A/4B site. The cleavage at the 3/4A junction of the precursor protein was difficult to observe since the processed ΔNS3 product with a predicted molecular mass 0.8 kDa was too small to be detected in this assay. As expected, processing was not observed in the absence of NS3 (lane 1). Minor bands observed in this autoradiogram are possibly due to incomplete synthesis of the full length substrate during *in vitro* translation. As in NS4A/4B, the processing of the precursor polypeptide containing the 5A/5B site by the NS3 alone was observed (Fig. 2, lane 8), and was much improved in the presence of the NS4A peptide (compare lanes 9 and 8). The two products of predicted molecular mass of 22.4 and 12 kDa correspond to the Δ5B and Δ5A proteins, respectively. The band representing the cleavage product ΔNS5A produced from ΔNS5A-ΔNS5B protein (lanes 8 and 9) was consistently observed to migrate slower than its expected molecular mass. High proline content in ΔNS5A (>16%) and the precursor protein ΔNS5A-ΔNS5B possibly affect the mobility in gel electrophoresis (Zakut-Houri *et al.,* 1983). We did not observe any detectable cleavage at the 4B/5A site of the precursor polypeptide (Δ4B/Δ5A) by NS3 alone (Fig. 2, lane 5). However, the polypeptide was cleaved to the expected products Δ4B and Δ5A when NS4A peptide was included in the cleavage reaction (Fig. 2, lane 6).

These results indicate that while the NS3 proteinase alone is sufficient for the cleavage at 4A/4B and 5A/5B sites, addition of NS4A is required for the cleavage at

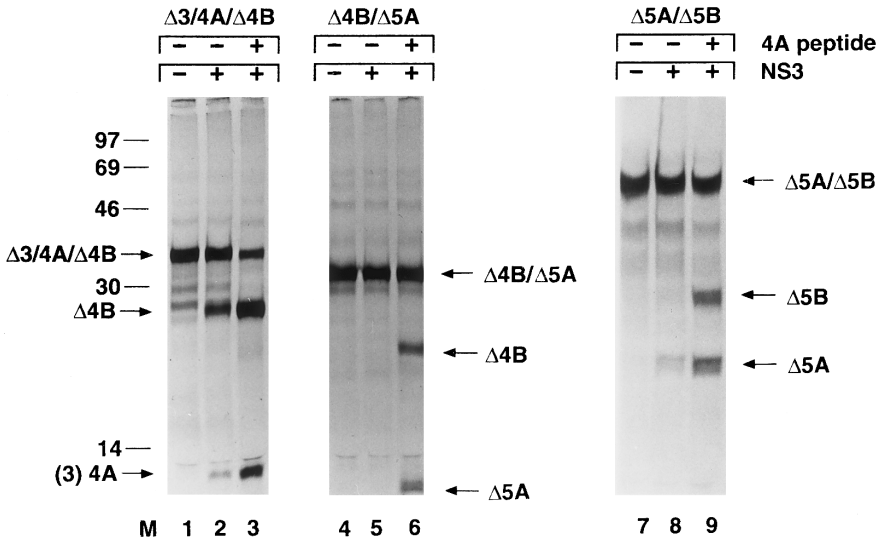

FIG. 2. HCV NS3 proteinase activity +/− 4A peptide on HCV polyprotein cleavage sites. ^{35}S-labeled substrates ΔNS3-4A-Δ4B (lanes 1–3), Δ4B-Δ5A (lanes 4–6), and Δ5A-Δ5B (lanes 7–9) were produced by *in vitro* translation in rabbit reticulocyte lysates as described under Materials and Methods. Labeled substrates and 1 μM purified NS3 proteinase were incubated in the presence or absence of 2 μM NS4A synthetic peptide [aa 22–54, HCV-1b (BK) strain] for 1 hr at 30°. The samples were analyzed on SDS–15% PAGE. HCV proteins are indicated by arrows. Molecular weight markers in kDa are shown on the left.

4B/5A site; NS4A increases the cleavage efficiency at 4A/4B and 5A/5B junctions. This is consistent with the results reported using transient expression of the HCV polyprotein in whole cells (Failla *et al.,* 1994, 1995; Lin *et al.,* 1994). The ability of the 33-aa peptide (aa 22–54) derived from NS4A sequence in HCV-1b (BK) to act as a cofactor for HCV-1a (FDA) NS3 proteinase indicates cross-reactivity between the two subtypes.

Mapping the minimum domain of NS4A for cofactor activity

The results described above clearly suggest that the carboxy-terminal 33-aa peptide of NS4A is a cofactor for the NS3-catalyzed cleavages in the nonstructural region of HCV polyprotein. To further characterize NS4A cofactor activity, additional truncations were made in the NS4A sequence of HCV-1a (FDA), as shown in Fig. 3. The truncated peptides were evaluated for their activity in a *trans*-cleavage reaction using the 5A/5B polypeptide substrate. We observed that in addition to the HCV-1b (BK) strain C-terminal 33-aa peptide, the HCV-1a (FDA) strain carboxy-end 33-aa peptide (aa 22–54) and an 18-aa peptide (aa 19–36) were able to enhance the NS3 proteinase activity (Fig. 4A, lanes 8, 9, and 10). In contrast, other NS4A peptides, including the amino-terminal 21-aa (aa 1–21) and two shorter truncations from the carboxyl end, a 15-mer (40–54 aa) and a 22-mer (aa 33–54), were found to have no detectable enhancement activity in this assay (lanes 5, 6, and 7, respectively). A non-HCV peptide of 18-aa was also found to be inactive (Fig. 4A, lane 4). The C-terminal 33-aa peptide (aa 22–54) from the 1a (FDA) strain was less active in our assay than its counterpart from the 1b (BK) strain (compare lanes 8 and 9, Fig. 4A). On peptide analysis (data not shown) we found that the cysteine content in the FDA peptide was rapidly decreased with time in DMSO, whereas the tyrosine content was unchanged, suggesting that the 2 terminal cysteine residues in the FDA peptide were oxidized to a less active form.

The central 18-aa peptide (aa 19–36) which appears to be sufficient for the NS4A cofactor activity in our biochemical assays was progressively truncated at both termini to determine the minimum region necessary for the NS4A cofactor activity. The various truncated forms were assessed for activity in the *in vitro* proteinase assay. The results are shown in Fig. 4B. We found that truncation of the 18-aa peptide (aa 19–36) to a 14-aa peptide (aa 21–34) and subsequently to a 13-mer (aa 22–34) did not significantly affect the cofactor activity in our assay (Fig. 4B, lanes 5 and 7). However, further deletion of the amino-terminal cysteine residues from the 13-mer (aa 22–34) peptide resulted in a loss of the activity (lane 10), implying that the cysteine residue is important for cofactor activity, and hence delineating the amino-terminal boundary of the domain. For the carboxy-end boundary, we found that the cofactor activity was retained until removal of the glycine residue (G_{33}) from the 13-mer (aa 22–34) peptide; this removal abolished the cofactor-enhanced cleavage activity within the limits of detection in our assay (compare lane 9 to lane 2). These results suggest that the 12-aa residue (aa 22 to 33) of NS4A constitutes the core sequence necessary to enhance the NS3-catalyzed proteolysis. Similarly, a 12-aa peptide (aa 22–

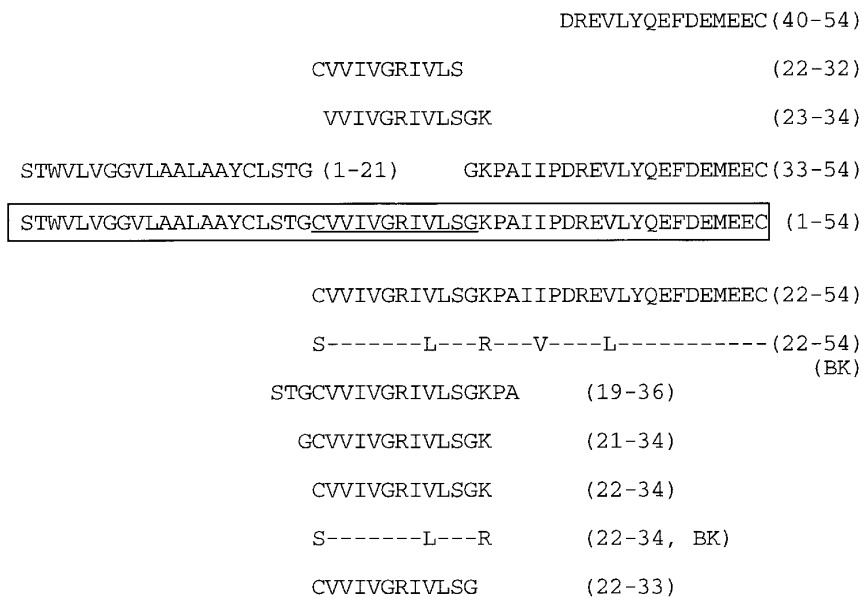

FIG. 3. Sequence of NS4A peptides used in these studies. Sequence of the complete NS4A protein is shown in the box, with the minimum sequence for cofactor activity underlined. The 4A peptides shown above the box had no activity; those peptides shown below the box retained cofactor activity. Dashed lines in BK-specific sequences represent identical amino acids within the FDA strain while the letters designate differences in the sequences.

33) containing the NS4A from HCV-1b (BK) was found to be the minimum domain needed to enhance NS3-mediated proteolysis. The same minimum sequence is sufficient for the NS3-mediated cleavage at 4B/5A site (N. J. Butkiewicz, unpublished data).

Requirement of NS4A for cleavage at the NS4A/4B site

The polypeptide substrate with the NS4A/4B site was observed to be cleaved by NS3 alone (Fig. 2, lane 2). This is possibly due to the fact that the NS4A cofactor sequence is present as a part of the polypeptide substrate. NS4A has been shown to activate the NS3 proteinase when fused to the substrate (Failla *et al.*, 1994) or coproduced with enzyme (Lin and Rice, 1995). Having identified the minimum domain of NS4A for the cofactor activity, we examined its role on the NS3-mediated cleavage at the NS4A/4B site by deleting the cofactor sequence from the polypeptide substrate. The modified ΔNS4A-ΔNS4B substrate (25.4 kDa) containing amino acid residues from 36 through 54 of NS4A and the amino-terminal 192 aa of NS4B was expressed in a cell-free translation system and processed with NS3 proteinase with and without the addition of the NS4A peptide. In the absence of the NS4A cofactor sequence, the modified NS4A-4B substrate was not cleaved by the NS3 proteinase (Fig. 5, lane 2); however, the substrate was readily cleaved when the 14-aa NS4A (aa 21–34) peptide was included in the reaction mixture (Fig. 5, lane 3). These results clearly demonstrate that NS4A cofactor activity is required for cleavage at the 4A/4B site by NS3 proteinase.

Identification of important residues in the NS4A peptide for cofactor activity

The ability of the 12-aa NS4A (aa 22–33) peptide to act as a cofactor in the NS3-mediated proteolysis prompted us to further characterize the underlying interaction by studying the importance of individual amino acids in the cofactor activity. We synthesized a series of NS4A peptide derivatives by sequentially substituting an alanine residue for each amino acid in the 13-mer peptide (aa 22–34) containing the NS4A 1a (FDA) sequence (Fig. 6A). The alanine-substituted peptides were evaluated for cofactor activity in *trans*-cleavage reactions using NS3 proteinase and polypeptide substrates ΔNS4A-Δ4B and ΔNS5A-Δ5B. The mutated peptides were tested at comparable concentrations (1.5 μM) as determined by amino acid analysis. Results are summarized in Figs. 6B and 6C. We found that the substitution of alanine for isoleucine 29 abolished the cleavage at both the 4A/4B and the 5A/5B sites within the limits of detection (lane 11, Figs. 6B and 6C); the cofactor activity was not recovered even using the peptide at five times higher concentration (data not shown). In addition to the substitution at I_{29}, we observed that the substitution of alanine for the amino acids V_{24}, I_{25}, and V_{26} caused a significant reduction of the NS4A cofactor activity as shown by decreased product formation (lanes 6, 7, and 8, respectively, Figs. 6B and 6C). Substitution with an alanine resi-

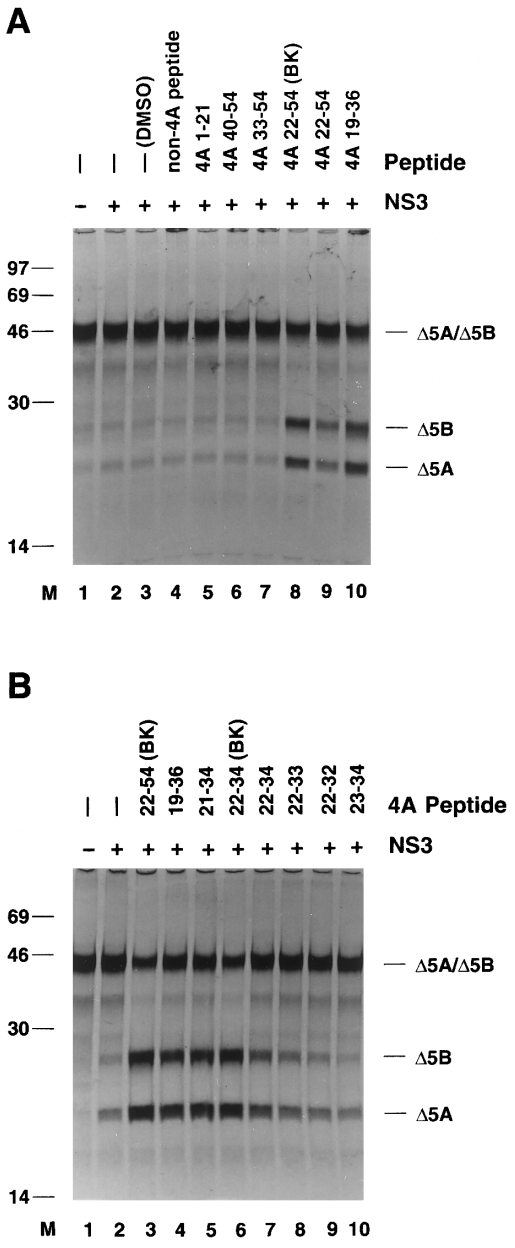

FIG. 4. Mapping the minimal NS4A domain required for enhancement of NS3 proteinase activity. ^{35}S-labeled ΔNS5A-Δ5B substrate was incubated with 1 μM purified NS3 proteinase in the presence of synthetic peptides from various NS4A sequences. HCV proteins are shown on the right; molecular weight markers in kDa are on the left. Unless indicated as BK (strain), all NS4A sequence is from HCV-1a (FDA) strain. (A) NS4A peptides were tested at 1 μM. A non-HCV NS4A 18-aa peptide (SNAEAGALVNASSAAHVD) was also tested. (B) NS4A peptides were tested at 2 μM.

due at amino acids V_{23}, G_{27}, V_{30}, and L_{31} was also found to impair the cofactor activity in our assay, particularly for NS5A/5B cleavage reaction (Fig. 6C, lanes 9, 12, and 13). Substitution outside the minimum domain (K34A) was observed to have marginal effect on cleavage at 5A/5B site only (Fig. 6C, lane 16). These results strongly suggest that the hydrophobic amino acid residues in the central region of NS4A, especially I_{29}, play critical roles in NS4A-mediated cofactor activity.

Complex formation between NS3 and the NS4A peptide and demonstration of the activity

Finally, we examined whether the peptide can form an enzymatically active complex with the proteinase. Taking advantage of the polyhistidine tag in the His-HIV-NS3 183 fusion protein, we used Ni-NTA beads to isolate the NS4A peptide–enzyme complex from free peptides. The complex was assayed in the 4A-dependent cleavage reaction using the ΔNS4A-Δ4B polypeptide substrate to show the presence of the NS4A. A 13-aa residue peptide (aa 22–34) and the mutant peptide with I25A and I29A substitutions were used in the experiment. Results are shown in Fig. 7A. Processing of the polypeptide substrate was observed by the imidazole-treated beads containing the native peptide and the enzyme (Fig. 7A, lane 5); cleavage activity was also observed, to a lesser extent, in the complex without imidazole elution (Fig. 7A, lane 2). None of the other bead preparations which were incubated with the NS3 alone (lanes 1 and 4) or with the mixture of NS3 and the mutated peptide (lanes 3 and 6) showed any proteinase activity. The results suggest that the 13-aa residue NS4A peptide interacts, possibly via specific hydrophobic residues, with the NS3 catalytic domain to form an enzymatically active complex. This is further supported by the fact that nonspecific association of the peptide with beads, if any, did not contribute toward co-

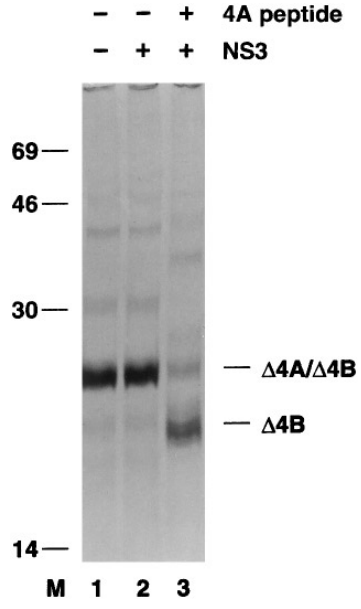

FIG. 5. Absolute requirement for NS4A in ΔNS4A-Δ4B cleavage. ^{35}S-labeled substrate ΔNS4A-Δ4B (aa 1693–1903), which does not contain the minimum cofactor domain of NS4A, was incubated with NS3 proteinase plus or minus 2 μM NS4A synthetic peptide (aa 21–34). HCV proteins are shown on the right; molecular markers in kDa are on the left.

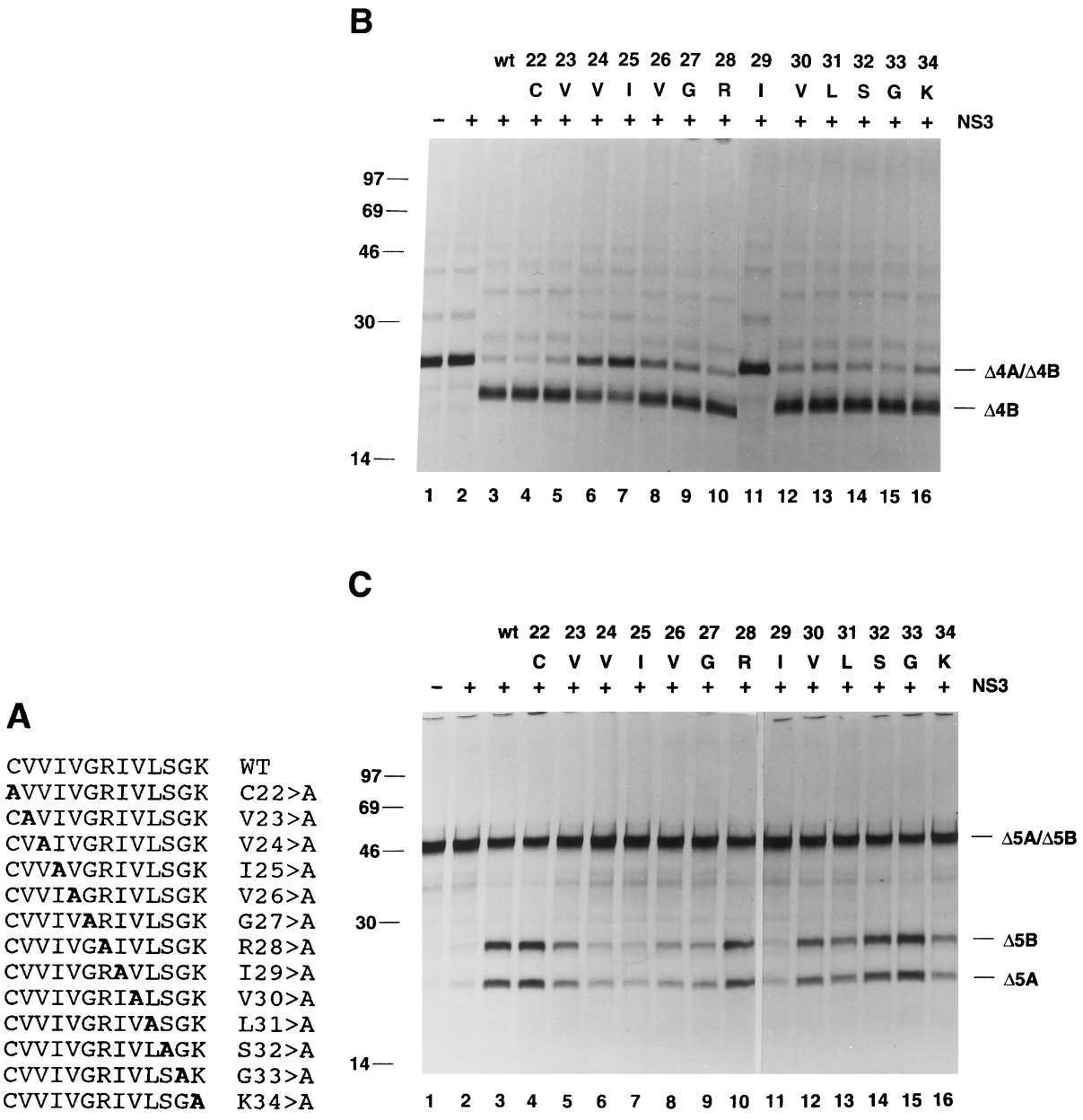

FIG. 6. Cofactor activity of alanine-substituted peptides derived from the 13-mer peptide (aa 22–34) of NS4A. (A) Wild-type (WT) alanine-substituted 4A peptides used in this study. Cofactor activity was tested at 1.5 μM on two substrates: ΔNS4A-Δ4B (B) and ΔNS5A-Δ5B (C). The alanine-substituted residue and the corresponding amino acid number in NS4A are shown above each lane. HCV proteins are shown on the right; molecular weight markers in kDa are on the left.

factor activity (Fig. 7B, lane 3). The cofactor activity of the NS4A peptide was only observed when the peptide was incubated with beads in the presence of NS3 (Fig. 7B, lane 2). As expected, the bead preparation without NS4A peptide did not show any protease activity (Fig. 7B, lane 1). These results clearly suggest that NS4A peptide specifically interacts with NS3 to form an enzymatically active complex.

DISCUSSION

In this report, we have used the purified NS3 proteinase catalytic domain and NS4A synthetic peptides to study the role of NS4A in NS3-catalyzed proteolysis of HCV nonstructural proteins in a cell-free biochemical assay. We show that the central hydrophobic region of NS4A (aa 22–33) is sufficient to act as a cofactor for NS3-mediated proteolysis at 4A/4B, 4B/5A, and 5A/5B sites. Previous studies using cell-free translation and cell culture transient expression of HCV polyprotein proposed a similar minimum functional domain for NS4A (Lin *et al.,* 1995; Bartenschlager *et al.,* 1995; Tanji *et al.,* 1995).

Since NS4A has been shown to enhance NS3 activity *in cis,* when fused with the substrate, or *in trans* (Failla

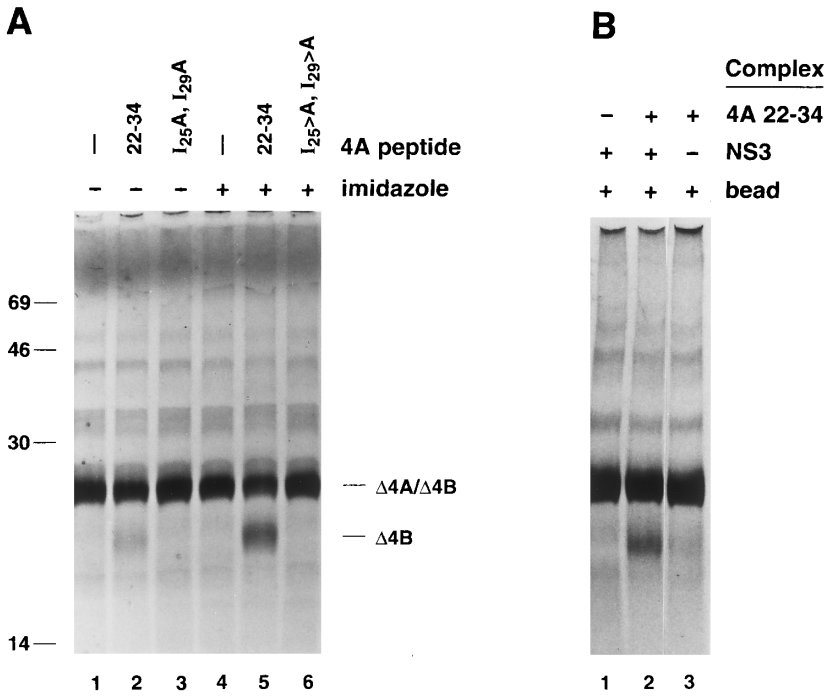

FIG. 7. Complex formation of NS3 proteinase with NS4A peptide. (A) Purified NS3 proteinase and NS4A synthetic peptide (aa 22–34), either native or double mutation ($I_{25}A$, $I_{29}A$), were allowed to interact together with Ni-NTA beads at 4° for 1 hr. Washed beads were tested for active complex formation by *in vitro* translation assay using ΔNS4A-Δ4B substrate, in the presence or absence of 150 m*M* imidazole. (B) An additional complex consisting of NS4A peptide (aa 22–34) and Ni-NTA beads was tested as control. Just before assay, 0.1 μM NS3 proteinase and 150 m*M* imidazole were added to the reactions.

et al., 1994; Bartenschlager *et al.,* 1994), the presence of the minimum functional region of NS4A sequence in 4A-4B polypeptide substrates warranted further characterization. By deleting the minimum functional sequence from the NS4A-4B polypeptide substrate, we have demonstrated that, like the proteolytic processing at 4B/5A junction (Fig. 2), the cleavage at the 4A/4B site requires the NS4A cofactor activity (Fig. 5). The requirement of the same functional domain of NS4A for all these *trans*-processing activities of NS3 indicates a common mechanism for NS4A even though the stringency of its functional requirements are different.

The significance of the NS4A central domain, particularly the hydrophobic amino acids, in NS3-mediated cleavages of HCV polyprotein has been reported in previous studies (Lin *et al.,* 1995; Bartenschlager *et al.,* 1995). Our experiments, which focused on further characterization of the peptide sequence in respect to the individual amino acid residues required for the cofactor activity, have identified isoleucine residue (I_{29}) as a critical amino acid for the 4A-dependent and 4A-augmented NS3 proteinase activity (Figs. 6B and 6C). This amino acid residue is also important for cofactor activity in processing at the 4B/5A junction, since its substitution to alanine abolished the cofactor activity of the peptide (N. J. Butkiewicz, unpublished results). The importance of I_{29} for the cofactor activity of full length NS4A has been demonstrated by Lin *et al.* (1995) in cell culture protein processing studies. Alanine substitution of other hydrophobic amino acids in the peptide (V_{24}, I_{25}, V_{26}, V_{30}, and L_{31}) inhibited the cofactor activity, whereas little or no effect was observed for C_{22} or R_{28}. Interestingly, deletion of either C_{22} in HCV-1a (FDA) or S_{22} in HCV-1b (BK) abolished cofactor activity of the peptides (Fig. 4B), indicating that an amino acid residue at this position may have a role in structural stabilization. From secondary structure predictions, this central region of NS4A exhibited a high propensity for the formation of a β-strand-like structure. Compatible with this prediction, it has been demonstrated from CD analysis that a synthetic peptide containing amino acid residues 22 to 33 of NS4A adopts a β-strand conformation in solution (W. Windsor, personal communication). It is possible that the local conformation of this region of NS4A is β-strand-like, and upon complex formation with the NS3 proteinase domain, favorable interactions are elicited, mainly through hydrophobic contacts from residues involving V_{24}, I_{25}, V_{26}, V_{30}, and L_{31}, and in particular I_{29}. Primary sequence analysis of different HCV strains corresponding to this central region of NS4A indicates that amino acid residues at positions I_{25}, V_{26}, and I_{29} are highly conserved, allowing for only very conservative substitutions to occur (e.g., V_{26}I, I_{29}V, L). In contrast, the substitution pattern of residues at positions 30 to 33 are much more diverse, with a high degree of toler-

ance for nonconservative substitutions in some cases (e.g., $V_{30}H$, E; $S_{32}N$, E; $G_{33}Q$, D). It should be noted that the 12-amino-acid residue peptide, aa 22–34 from HCV-NZL1 strain (CVVIVGHIEIEGK), was found to enhance the cleavage at 5A/5B junction in our assay (Butkiewicz *et al.,* unpublished results). While this manuscript was being prepared, Shimuzu *et al.* (1996), using various substitutions in the NS4A peptide, observed that amino acid residues V_{23}, I_{25}, G_{27}, R_{28}, I_{29}, and L_{31} were important for enhancing the cleavage of NS 5A-5B synthetic peptide substrate by the full length NS3 proteinase. Except for R_{28}, the findings are in agreement with our observations. Interestingly, the observation that substitution of the arginine residue with a serine residue is less deleterious for cofactor function than with either aspartic acid or glutamine (Shimuzu *et al.,* 1996) suggests that a neutral or weakly hydrophobic amino acid is better tolerated in that position. This is supported by our findings that $R_{28}A$ substitution did not significantly affect the cofactor activity.

The mechanism by which NS4A activates NS3-mediated proteolysis of HCV nonstructural protein is not clear. NS4A is cleaved off from the proteinase by an intramolecular cleavage and has been shown to activate the proteinase *in cis* or *in trans* (Failla *et al.,* 1994; Lin *et al.,* 1994). Liberation of NS4A from polypeptide substrates is not a prerequisite for its activity (Failla *et al.,* 1994). NS4A can activate NS3 proteinase even when it is covalently fused to the proteinase domain (Hahm *et al.,* 1995; Butkiewicz and DasMahapatra, unpublished results). It has been suggested that, like the NS2B/3 proteinase in flaviviruses (Arias *et al.,* 1993; Chambers *et al.,* 1991; Falgout *et al.,* 1993), NS4A-assisted cleavage reactions in HCV are carried out by a proteinase complex consisting of NS3 and NS4A. Although the full length NS4A has not been used in our binding experiments, the ability of the 13-aa cofactor peptide to form an enzymatically active complex with the proteinase domain suggests that active proteinase is a heterodimeric complex between NS3 and NS4A. Complex formation between NS3 and NS4A has been suggested based on their ability to form an immunoprecipitable complex in transfected cells (Lin *et al.,* 1995; Bartenschlager *et al.,* 1995). Based on coimmunoprecipitation results using various NS4A mutants, Lin *et al.* (1995) suggested that NS4A central region (aa 21–34) was important for the interaction. It is possible that the association of the 13-amino-acid NS4A peptide with NS3 may induce conformational changes favoring substrate hydrolysis. In a recent report (Steinkuhler *et al.,* 1996), structural rearrangement in the substrate binding pocket of NS3 proteinase has been suggested based on the kinetic data. It is worth mentioning that in our experiments we observed no significant difference in the cofactor activity among the 14-mer (aa 21–34) and the 18-mer (aa 19–36) peptides containing sequence of HCV-1a (FDA) (Fig. 4B, lanes 5 and 4), and among the 13-mer (aa 22–34) and the 33-mer (aa 22–54) of HCV-1b (BK) peptides (Fig. 4, lanes 6 and 3). This is in agreement with the reported data (Steinkuhler *et al.,* 1996), where two peptides (Pep4a21-34 and Pep4A21-54) were indistinguishable in activating NS3. These results indicate that the minimal activating sequence of NS4A is sufficient for interaction with NS3, although full length NS4A, which is involved in multiple interactions with NS3, is possibly required for stabilizing the complex and/or for membrane association.

Specific proteolysis to produce mature viral products is critical for replication of many viruses, and regulation of these specific events to prevent inappropriate proteolysis is obviously vital. Viruses employ various mechanisms to modulate this important function. In HIV-1, dimerization is required for activation of the proteinase (Navia *et al.,* 1989); also, Krausslich *et al.* (1991) showed that premature activation of the HIV-1 protease is toxic to cells and prevents virus assembly. Ribosomal frameshifting to produce gag–pol fusion protein containing the protease is possibly used to limit the amount of protease synthesis (Jacks *et al.,* 1988). In adenovirus, a heterodimeric complex formation between an 11-amino-acid residue virus-encoded peptide and the protease is required for enzyme activation (Webster *et al.,* 1993; Mangel *et al.,* 1993). For picornaviruses, the proteolytic cleavages within the capsid precursor P1 are catalyzed by a complex containing the 3C proteinase and 3D polymerase (Ypma-Wong *et al.,* 1988; Jore *et al.,* 1988). It is noted that several other viral proteases, including flavivirus NS3 (Rice, 1995) and Sindbis virus nsp2 (de Groot *et al.,* 1990), require a cofactor to catalyze proteolysis. It is possible that HCV may adopt a similar strategy to coordinate the regulation of its nonstructural protein processing to mature products by modulating the NS3 proteinase activity. Understanding the nature of the interaction between the NS3 proteinase and the NS4A cofactor will provide an attractive alternative target for the development of specific proteinase inhibitors as clinically viable antiviral agents.

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